Expression of the melanoma cell adhesion molecule in human mesenchymal stromal cells regulates proliferation, differentiation, and maintenance of hematopoietic stem and progenitor cells

Sabine Stopp,[1] Martin Bornhäuser,[1,2] Fernando Ugarte,[3] Manja Wobus,[1] Matthias Kuhn,[4] Sebastian Brenner,[2,3]* and Sebastian Thieme[3]*

[1]Medical Clinic and Policlinic I; [2]Center for Regenerative Therapies, Dresden; [3]Department of Pediatrics, University Hospital "Carl Gustav Carus" Dresden; and [4]Institute for Medical Informatics and Biometry, Technical University Dresden, Germany

ABSTRACT

The melanoma cell adhesion molecule defines mesenchymal stromal cells in the human bone marrow that regenerate bone and establish a hematopoietic microenvironment *in vivo*. The role of the melanoma cell adhesion molecule in primary human mesenchymal stromal cells and the maintenance of hematopoietic stem and progenitor cells during *ex vivo* culture has not yet been demonstrated. We applied RNA interference or ectopic overexpression of the melanoma cell adhesion molecule in human mesenchymal stromal cells to evaluate the effect of the melanoma cell adhesion molecule on their proliferation and differentiation as well as its influence on co-cultivated hematopoietic stem and progenitor cells. Knockdown and overexpression of the melanoma cell adhesion molecule affected several characteristics of human mesenchymal stromal cells related to osteogenic differentiation, proliferation, and migration. Furthermore, knockdown of the melanoma cell adhesion molecule in human mesenchymal stromal cells stimulated the proliferation of hematopoietic stem and progenitor cells, and strongly reduced the formation of long-term culture-initiating cells. In contrast, melanoma cell adhesion molecule-overexpressing human mesenchymal stromal cells provided a supportive microenvironment for hematopoietic stem and progenitor cells. Expression of the melanoma cell adhesion molecule increased the adhesion of hematopoietic stem and progenitor cells to human mesenchymal stromal cells and their migration beneath the monolayer of human mesenchymal stromal cells. Our results demonstrate that the expression of the melanoma cell adhesion molecule in human mesenchymal stromal cells determines their fate and regulates the maintenance of hematopoietic stem and progenitor cells through direct cell-cell contact.

Introduction

Multipotent human mesenchymal stromal cells (hMSC) support the growth of hematopoietic stem and progenitor cells (HSPC) during *ex vivo* co-culture.[1-3] hMSC produce various growth factors, adhesion molecules, and matrix proteins contributing to the formation of stem cell niches, thereby controlling the homing, maintenance, and differentiation of HSPC.[1,4] Well-studied signaling pathways within this niche include Notch, Wnt, and Hedgehog.[5,6] Soluble signaling molecules include cytokines, growth factors, or chemokines (e.g. stem cell factor (SCF), the FLT3 ligand (FLT3-L), and stromal derived factor-1 (SDF-1)).[7,8] It is believed that direct cell-cell contact mediated by adhesion molecules is essential for the maintenance of immature HSPC. Several adhesion molecules (VCAM-1, ICAM-1, N-cadherin, and NCAM) are known to be important for niche formation[4,6-8] and hematopoiesis.

The melanoma cell adhesion molecule (MCAM/CD146) is used as a marker for mesenchymal stromal cells. In a xenotransplantation model, Sacchetti *et al*. demonstrated that culture-expanded MCAM$^+$ bone marrow stromal cells reconstituted the hematopoietic microenvironment.[9] Furthermore, the expression pattern of MCAM on bone marrow-derived mesenchymal stromal cells correlated with their *in situ* localization.[10] However, the exact function of MCAM within the human bone marrow niche is unclear. MCAM is a 113 kDa glycoprotein that belongs to the immunoglobulin (Ig) superfamily of cell adhesion molecules. It contains an extracellular domain with five Ig domains (V-V-C2-C2-C2), a transmembrane domain, and a cytoplasmic domain with potential recognition sequences for protein kinases.[11] MCAM orthologs have been identified in mouse, rat, chicken, and zebrafish.[12] Human MCAM was originally identified as a marker for melanoma progression and metastasis. MCAM is further expressed by the vascular endothelium, smooth muscle cells, activated T lymphocytes, and bone marrow stromal cells.[11] MCAM function has been extensively studied in melanomas and other types of cancer (prostate cancer and breast cancer), but the ligand for MCAM has not yet been identified.[12-14]

This study aimed to clarify the impact of MCAM expression on the functional properties of hMSC and the maintenance of HSPC in co-culture. Thus, we generated primary hMSC that stably expressed shRNA against MCAM or that over-expressed an MCAM coding sequence (CDS) through lentiviral vector gene transfer. Our findings indicate that MCAM expression has a pivotal role in hMSC differentiation and the maintenance of HSPC through direct cell-cell contact.

Design and Methods

Isolation of hMSC and HSPC

Primary hMSC and HSPC were isolated from healthy donors after informed consent and the approval of the local ethics committee. Primary hMSC were isolated from bone marrow aspirates, as

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Correspondence: sebastian.thieme@uniklinikum-dresden.de

described previously.[15] CD34+ HSPC were purified from either mobilized peripheral blood or umbilical cord blood using CD34 antibody-conjugated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. The purity of hMSC and HSPC cells was evaluated by flow cytometry (hMSC: CD45−, CD34−, CD73+, CD90+, CD105+, CD166+; HSPC: CD45+, CD34+, CD133+, CD38−/dim). hMSCs were cultured in DMEM GlutaMax (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FCS; Biochrom, Cambridge, UK). All experiments were performed with hMSC that were passaged only once to avoid any variations due to the aging of cells. Isolated HSPCs were cultured in corresponding media.

Lentiviral vectors, virus vector production, and hMSC transduction

Plasmid pLKO.1 vectors encoding shRNAs that targeted human MCAM (KD1 and KD2) were obtained from OpenBiosystems (Huntsville, AL, USA). Control pLKO.1 vectors (empty vector control or vector with shRNA targeting eGFP) were obtained from Sigma-Aldrich (Taufkirchen, Germany).

The MCAM coding sequence was amplified from a cDNA library and directionally cloned into the lentiviral vector pRRL.SIN.cPPT.SFFV.GFP.WPRE[16,17] (kindly provided by Christopher Baum, Hannover, Germany) fused with an IRES-GFP sequence (MCAM-GFP) using XhoI/BamHI restriction sites. To produce lentiviral vector particles, HEK293T cells were transfected with lentiviral vectors in combination with the packaging plasmids psPAX and pVSVg using PEI.[18] Lentivirus-vector containing media were collected 48 h after transfection. Primary hMSC were infected three times with lentiviral vector particles (0.5 x viral supernatant) in the presence of 1 μg mL^{-1} protamine every 12 h.

Assessment of cell differentiation, proliferation, β-galactosidase staining and migration of transduced hMSC

Twelve hours after seeding the transduced hMSC, the medium was replaced with differentiation medium. Osteogenic differentiation medium was supplemented with 10 mM β-glycerophosphate, 5 μM dexamethasone, and 200 μM ascorbic acid (Sigma-Aldrich). Mineralization was analyzed with von Kossa staining ten days after differentiation. In brief, cells were washed once with phosphate-buffered saline (PBS), fixed with 3.7% formaldehyde, washed, stained with 1% $AgNO_3$, and developed using 0.1% pyrogallol. Alkaline phosphatase activity was measured in cell extracts using the pNPP Liquid Substrate System (Sigma-Aldrich). The adipogenic differentiation medium contained 50 μM dexamethasone, 500 μM 3-isobutyl-1-methylxanthine, 1 mg mL^{-1} insulin, and 100 μM indomethacin (Sigma-Aldrich). Adipocytes were quantified after 14 days using Oil Red staining. In brief, cells were washed with PBS, fixed with 4% paraformaldehyde, washed, and stained with Oil Red solution for 15 min at room temperature. Excess stain was removed using two washes with 60% ethanol. Adipocytes were visualized using a microscope (Zeiss Axiovert 400M; 100x magnification). Cell proliferation was measured using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Roche, Mannheim, Germany) assay. In brief, 1000 cells/96-wells were seeded in five replicates and grown for 96 h. MTT was added to prepare a final concentration of 0.5 mg mL^{-1}. Formazan crystals were solubilized overnight and the absorbance was measured at 570 nm. For the EdU (5-ethynyl-2'-deoxyuridine) incorporation assay (Invitrogen), 1.5x10^5 cells were seeded in a T25 flask. Twelve hours after seeding, EdU was added to the cell culture medium to prepare a final concentration of 10 μM. After 24 h incubation, cells were harvested and analyzed by flow cytometry, according to the manufacturer's instructions. Cells without EdU incorporation were used as negative control. Cell cycle analysis was measured simultaneously with EdU incorporation by 7-AAD-staining. To detect senescent cells, 1x10^4 cells/24-well were seeded in duplicate for β-galactosidase staining (Cell Signaling Technology, Boston, MA, USA). Twenty-four hours after seeding, cells were subjected to a β-galactosidase assay, according to the manufacturer's instructions.

In vitro wounding assays were performed using 6-well plates to assess cell migration. Confluent hMSC monolayer were wounded with sterile 1000 μL pipette tips, washed twice with PBS to remove non-adherent cells, and incubated further in complete growth medium. Wounds were imaged at 0 h and after 20 h using phase-contrast microscopy. To mimic constitutive RhoA activation, 10 μM Nocodazole (Sigma Aldrich) was added to cell culture for 20 h. Cell migration was quantified using the ImageJ program (NIH, Bethesda, MD, USA).

Statistical analysis

Statistical analysis was performed using paired Student t-tests. The Cochran-Mantel-Haenszel test was used to assess qualitative differences in the formation of secondary colonies. Statistical significance was tested at $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. All results were expressed as mean±standard error of the mean (SEM) for at least 3 donors.

Results

Generation and characterization of MCAM knockdown or MCAM overexpression in primary hMSC

To analyze the role of MCAM within the bone marrow niche *in vitro*, we used lentiviral vector gene transfer to establish primary hMSC that stably expressed shRNAs against MCAM or that overexpressed MCAM CDS (*Online Supplementary Figure S1*). Protein and mRNA levels were analyzed to validate MCAM knockdown or overexpression. As shown in *Online Supplementary Figure S1A*, immunoblotting of whole cell lysates indicated a reduction in the MCAM protein level to 36% or 52% for two MCAM specific shRNAs, MCAM-KD1 and MCAM-KD2, respectively, compared with cells expressing control shRNA against eGFP or the empty pLKO.1 vector. Upon MCAM overexpression, the relative protein level was up-regulated 10.5-fold. Flow cytometric analysis of MCAM surface expression confirmed these results (*Online Supplementary Figure S1B and C*). Compared with pLKO.1 control, the average downregulation of MCAM expression after MCAM KD was 65% according to the mean fluorescence intensity (MFI). MCAM overexpression showed an average 8.2-fold upregulation in comparison to control (*Online Supplementary Figure S1E*). MCAM mRNA levels were similarly reduced to 24% and 34% for MCAM-KD1 and MCAM-KD2, respectively, compared to control. In contrast, MCAM overexpression strongly increased the mRNA level (130-fold; *Online Supplementary Figure S1D*). Because of the stronger knockdown, MCAM-KD1 was used in further experiments where it is referred to as MCAM-KD. The expression of typical hMSC surface markers (CD73, CD90, CD105, CD166) was unchanged after knockdown or overexpression of MCAM (*data not shown*).

Subsequently, we characterized the effects of MCAM knockdown and overexpression on the differentiation potential of osteogenic and adipogenic lineages (Figure 1A-C). von Kossa staining and ALP activity measurements

showed that MCAM knockdown in hMSC resulted in a significantly reduced potential of hMSC in differentiating into the osteogenic lineage (ALP: MCAM-KD 56.8±30.3 mU mg^{-1} *vs.* pLKO.1 175.8±58.3 mU mg^{-1}; $P<0.05$; Figure 1A and B). Interestingly, the potential for adipogenic differentiation was also significantly decreased by MCAM knockdown compared with the control cells (MCAM-KD 1.6±0.45% *vs.* pLKO.1 5.7±1.4% adipogenic differentiation; $P<0.05$; Figure 1A and C). In contrast, MCAM-overexpressing hMSC exhibited a significantly increased osteogenic differentiation compared with their respective control cells (ALP: MCAM-GFP 344.8±118.9 mU mg^{-1} *vs.* IRES-GFP 272.1±106.4 mU mg^{-1}; $P<0.05$; Figure 1A and B). However, adipogenic differentiation was not affected by MCAM overexpression (MCAM-GFP 6.8±2.7% *vs.* IRES-GFP 7.3±2.65% adipogenic differentiation; Figure 1A and C).

We further examined the proliferative behavior of hMSC after MCAM knockdown or overexpression using an MTT assay (Figure 1D) and by EdU incorporation (Figure 1E). MCAM knockdown resulted in a significant 2.3-fold decrease in metabolic activity and a 5.3-fold lower DNA synthesis rate compared with the pLKO.1-control. In contrast, MCAM-overexpressing hMSC showed an enhanced metabolic activity (1.4-fold) and an increased DNA synthesis rate (1.4-fold) compared with the IRES-GFP-control-transduced hMSC.

To analyze the impact of the varying proliferative capacity of transduced hMSC on the support of HSPC during co-culture we seeded equal numbers of hMSC as confluent layer. Counting hMSC numbers five days and four weeks after plating, no significant differences in cell numbers were observed between MCAM-KD and MCAM-GFP transduced hMSC, suggesting that contact inhibition prevented overgrowth of MCAM-GFP hMSC (*data not shown*).

Cell cycle analysis indicated cell cycle arrest during G1/S phase after MCAM knockdown (*data not shown*). Furthermore, p21 expression was up-regulated 2.9-fold in MCAM-KD hMSC compared with pLKO.1-control cells, whereas the p21 mRNA level was down-regulated 2.1-fold in MCAM-overexpressing hMSC (Figure 1F). Furthermore, the expression of CDK2 and Cyclin E (both promote G1/S phase cell cycle progression) were decreased upon MCAM knockdown. Also, the Rb (Retinoblastoma) protein was almost completely dephosphorylated upon MCAM knockdown compared with pLKO.1-control cells (Figure 1G). To confirm the impaired proliferation capacity upon MCAM knockdown, PCNA (proliferating cell nuclear antigen) was evaluated showing a decreased PCNA expression. In contrast, MCAM overexpressing hMSC exhibited an increased CDK2, Cyclin E and PCNA expression as well as an elevated phosphorylation of the Rb protein. The p53 expression level was not altered in hMSC with both MCAM-KD and MCAM overexpression (*data not shown*). Cells were stained to determine β-galactosidase accumulation, which is known to be a senescence marker, to test whether MCAM knockdown cells assumed a senescent-like phenotype.[19] hMSC with MCAM knockdown were highly positive for senescence associated (SA)-β-galactosidase staining (*data not shown*).

MCAM expression influences the cell motility in melanoma and endothelial cells,[20,21] so we further analyzed the migratory potential of hMSC after MCAM knockdown or overexpression. As shown in Figure 1H, MCAM knockdown led to impaired migration, whereas MCAM-overexpressing hMSC exhibited enhanced migration during *in vitro* wounding assays.

MCAM expression by hMSC maintains $CD34^+CD133^+$ HSPC

We used an MSC–HSPC co-culture system to determine whether MCAM expression on hMSC supports HSPC during *ex vivo* expansion.[22] Freshly isolated HSPC from G-CSF mobilized leukapheresis products were co-cultured on established MCAM-KD or MCAM-overexpressing hMSC monolayer for five days. To characterize the HSPC in more detail, the expression levels of CD34 and CD133 (characteristics of immature HSPC) as well as markers for early myeloid (CD13, CD33) and lymphoid (CD10, CD56) lineages were analyzed by flow cytometry (Figure 2). As shown in Figure 2A, co-culture of $CD45^+$ HSPC on an hMSC monolayer with MCAM knockdown resulted in a significant decrease in CD34 expression. A significant reduction in the proportion of $CD45^+CD34^+CD133^+$ cells was also observed. In contrast, after five days of *ex vivo* co-culture with MCAM-overexpressing hMSC, HSPC maintained a significantly higher CD34 expression and a higher proportion of $CD34^+CD133^+$ cells compared with co-cultures including IRES-GFP-control vector transduced hMSC (Figure 2B; Table 1). In line with these results, the percentage of more mature hematopoietic cell subsets (myeloid: $CD34^-CD33^+$ and $CD34^-CD13^+$; lymphoid: $CD34^-CD10^+$ and $CD34^-CD56^+$) was higher after five days of *ex vivo* co-culture with MCAM knockdown hMSC compared with pLKO.1-control (Figure 2C; Table 1), whereas co-culture with MCAM-overexpressing hMSC showed significantly reduced proportions of more mature cell subsets (Figure 2D; Table 1). When using non-contact conditions, we found no difference in the CD34 expression on HSPC or in the proportion of $CD34^+CD133^+$ HSPC with MCAM-KD or MCAM-overexpressing hMSC compared with their respective controls (*data not shown*). These data suggest that the maintenance of HSPC was mediated by direct cell-cell contact. HSPC co-cultured on hMSC with MCAM knockdown lost their immature phenotypes more rapidly than HSPC cultured on pLKO.1-control transduced hMSC. Therefore, we further tested whether MCAM expression by hMSC also affected the proliferation of HSPC. Thus, the cell division history of HSPC was tracked during co-culture using CFSE staining. As shown in Figure 2E, HSPC maintained for five days on MCAM knockdown hMSC demonstrated a significantly higher proliferation rate than HSPC maintained on pLKO.1-control transduced hMSC (cell division peak: MCAM-KD 7 *vs.* pLKO.1 6). The increased cell proliferation was also associated with a simultaneous loss of CD34 expression. In contrast, MCAM overexpression resulted in the opposite effect (cell division peak: MCAM-GFP 5 *vs.* IRES-GFP 6; Figure 2F).

To determine whether HSPC displayed different behavior of adhesion to MCAM-KD or MCAM-overexpressing hMSC, the number of attached cells was counted 24 h after seeding (*Online Supplementary Figure S2*). Co-culture with MCAM-KD hMSC led to a reduced number of adherent HSPC on the monolayer compared with the pLKO.1-control transduced monolayer ($5.2±1.6x10^4$ *vs.* $7.6±2x10^4$ cells mL^{-1}; $P<0.05$), whereas MCAM-overexpressing hMSC provided greater support for HSPC adhesion to the hMSC monolayer compared with the control

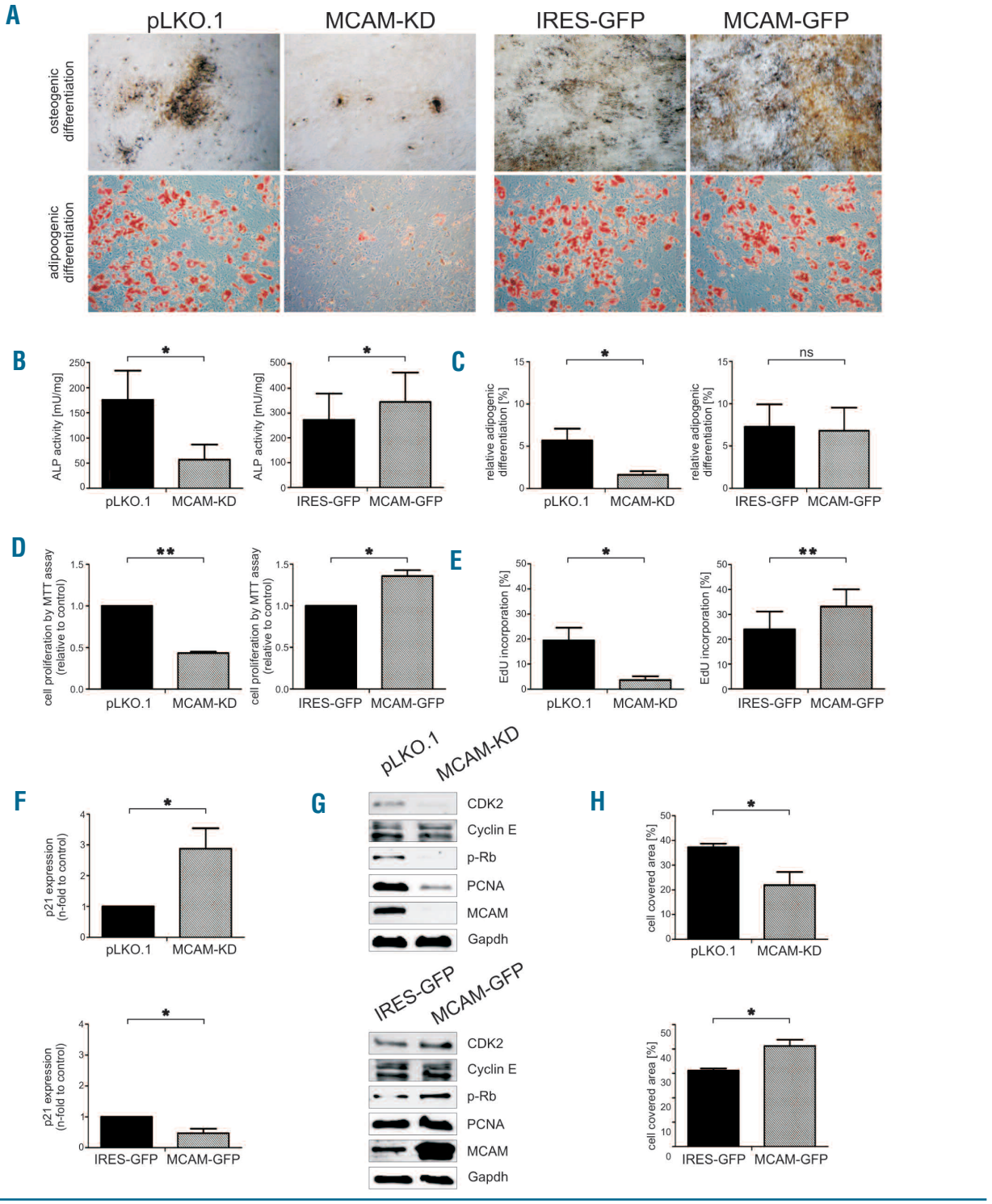

Figure 1. MCAM expression in hMSC enhances osteogenic differentiation, cell proliferation, and migration. (A) MCAM knockdown or overexpressing hMSC were cultured in osteogenic or adipogenic differentiation medium for 10 and 14 days, respectively. Osteogenic differentiation was monitored by von Kossa staining (Zeiss, Axiovert 400M; magnification 200x) and adipogenic differentiation by Oil Red staining (magnification 100x). (B) Osteogenic differentiation was quantified based on ALP activity 10 days after induction. Mean±SEM of at least 3 donors is shown (*P<0.05; paired Student's t-test). (C) Adipogenic differentiation was quantified based on Oil Red positive cells after 14 days of differentiation using in-house quantification software. Mean±SEM of at least 3 donors is shown (*P<0.05; paired Student's t-test). Cell proliferation of MCAM knockdown or MCAM-overexpressing hMSC was assessed by MTT assay and EdU incorporation. (D) In the MTT assay, hMSC expressing MCAM shRNA or MCAM CDS were grown for 96 h. Proliferation was normalized relative to the respective control cells (pLKO.1 *vs.* MCAM-KD; IRES-GFP *vs.* MCAM-GFP; paired Student's t-test). (E) In the EdU incorporation assay, cells were labeled with 10 μM EdU for 24 h and analyzed by flow cytometry. Mean±SEM for at least 3 donors is shown (*P<0.05; **P<0.01; paired Student's t-test). (F) Quantitative RT-PCR of the p21 mRNA levels. Results are normalized relative to the respective control cells (pLKO.1 *vs.* MCAM-KD; IRES-GFP *vs.* MCAM-GFP). Mean±SEM for at least 3 donors is shown (*P<0.05; paired Student's t-test). (G) Expression of cell-cycle regulators CDK2, Cyclin E and PCNA, as well as phosphorylation status of retinoblastoma (Rb) were assessed by immunoblotting in whole cell lysates of MCAM knockdown or MCAM-overexpressing hMSC and respective control cells (pLKO.1; IRES-GFP). Gapdh expression was used as loading control. (H) Migration of hMSC expressing MCAM-targeting shRNA, MCAM CDS, or the respective control vectors (pLKO.1 *vs.* MCAM-KD; IRES-GFP *vs.* MCAM-GFP) were subjected to *in vitro* wounding assays. Wounds were quantified after 20 h using ImageJ software. Mean±SEM of 5 donors is shown (*P<0.05; paired Student's t-test).

(11.9±3.8 x 10^4 *vs.* 9.3±3.4x10^4 cells mL^{-1}; *P*<0.05). In that context, we analyzed the surface expression of CD29 (β1-integrin) and found CD29 to be up-regulated upon MCAM knockdown (1.3-fold) and down-regulated upon MCAM overexpression (1.9-fold; *Online Supplementary Figure S2B*). However, the adhesion behavior of HSPC to MCAM-KD or MCAM-GFP hMSC does not depend on CD29 expression. Blocking of CD29 and another integrin, CD49e (α5-integrin) using neutralizing antibodies did not affect the adhesion of HSPC to MCAM-KD or MCAM-GFP hMSC (*data not shown*). In agreement with the study by Guezguez *et al.*,[14] MCAM better supported the migration of HSPC beneath the hMSC monolayer compared with the control transduced cells (MCAM-GFP 29.2±5.1% *vs.* IRES-GFP 22.5±4.1% *P*<0.05; *Online Supplementary Figure S2B*), whereas MCAM knockdown hMSC showed

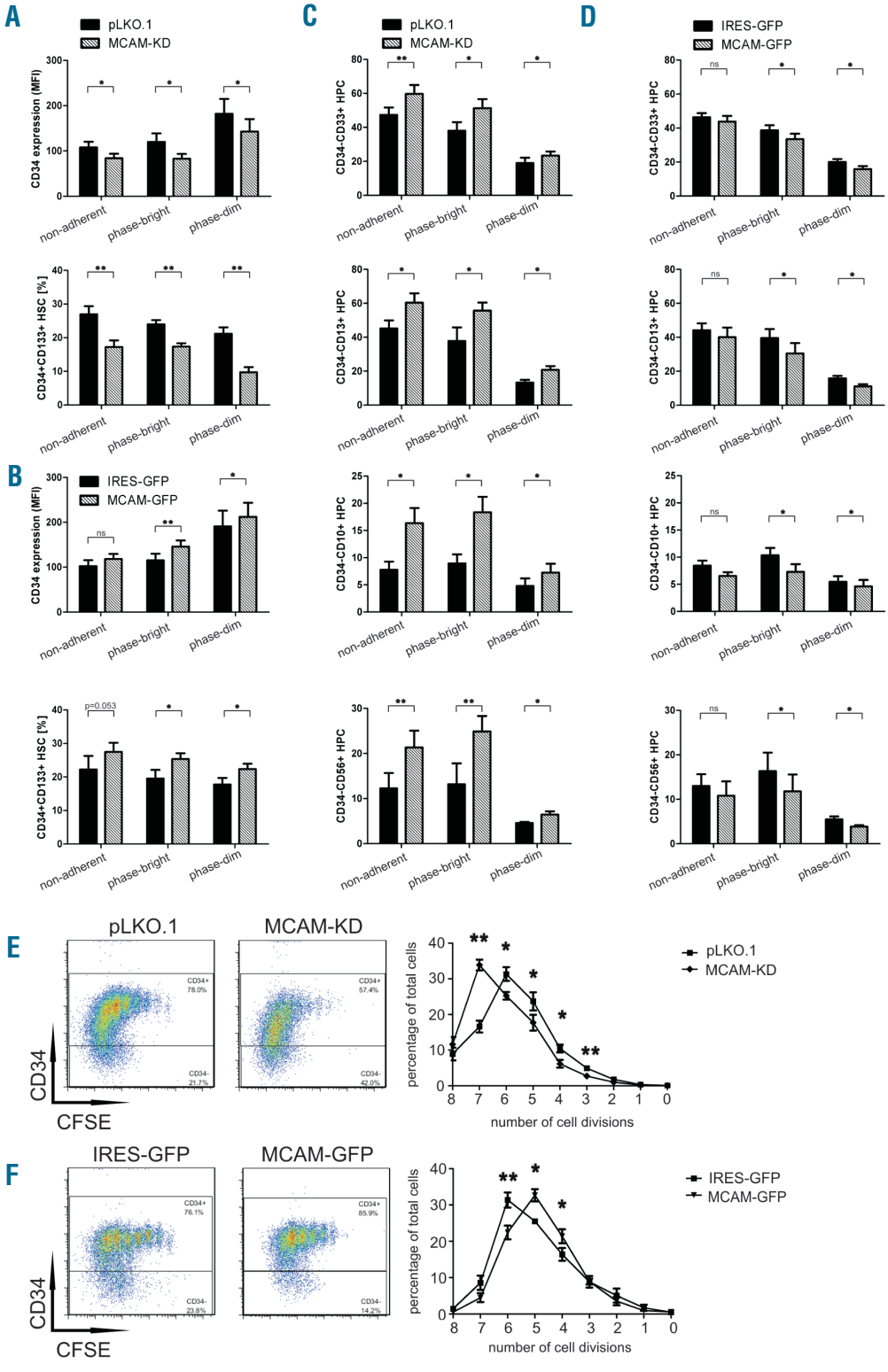

Figure 2. MCAM expression on hMSC supports $CD34^+CD133^+$ HSPC during *ex vivo* co-culture. (A) HSPC from leukapheresis were co-cultured on MCAM knockdown or (B) MCAM-overexpressing hMSC monolayer for five days and analyzed by flow cytometry to determine the mean fluorescence intensity (MFI) of CD34 expression in $CD45^+$ HSPC and the proportion of $CD45^+CD34^+CD133^+$ HSPC. Graphs show the mean±SEM for at least 5 donors (**P*<0.05; ***P*<0.01; paired Student's t-test). (C) HSPC from leukapheresis were co-cultured on MCAM knockdown or (D) MCAM-overexpressing hMSC as described in (A) and the proportion of $CD45^+CD34^-CD33^+$, $CD45^+CD34^-CD13^+$, $CD45^+CD34^-CD10^+$ and $CD45^+CD34^-CD56^+$ HSPC was analyzed by flow cytometry. Graphs show the mean±SEM of 4 donors (**P*<0.05; paired Student's t-test). (E) Representative FACS plots of CFSE-tracked HSPC co-cultured on MCAM knockdown or (F) MCAM overexpressing hMSC and co-stained for CD34. Graphs show the percentage of total cells in each cell division cycle calculated as mean±SEM for at least 3 donors (**P*<0.05, ***P*<0.01; paired Student's t-test).

less support for transmigration of HSPC beneath the hMSC monolayer (18.1±2.9% *vs.* 23.4±4.2%, $P<0.05$).

SDF-1 expression and secretion were analyzed to determine whether SDF-1 contributed to the observed phenotype. However, we observed no significant differences in SDF-1 expression or secretion after MCAM knockdown or overexpression (*data not shown*).

MCAM supports the maintenance of long-term culture-initiating cells

Long-term culture-initiating cell (LTC-IC) assays were performed to further understand the role of MCAM expression on hMSC for the maintenance of HSPC (Figure 3). HSPC from umbilical cord blood were cultured on MCAM knockdown or MCAM-overexpressing hMSC monolayer for four weeks. The total number of cobblestone-area forming cells (CAFC) was decreased 5.4-fold on MCAM knockdown monolayer compared with pLKO.1-control transduced monolayer (6.6±1.5 *vs.* 35.5±6.3; $P<0.001$; Figure 3A). MCAM-overexpressing hMSC promoted a 1.7-fold increase in CAFCs compared with CAFCs on the IRES-GFP-control transduced monolayer (63.8±10.2 *vs.* 36.6±4.9; $P<0.01$; Figure 3A). After four weeks of co-culture, we transferred CAFC-derived HSPC into semi-solid medium to quantify their capacity in secondary colony-forming cell assays (CFU; Figure 3B). We found a 2.1-fold lower CFU frequency (1.8%±0.5% *vs.* 3.8%±1.3%; $P<0.05$) in co-cultures with MCAM knockdown monolayer and a 1.6-fold higher CFU frequency (5.7%±1.5% *vs.* 3.8%±1.4%; $P<0.01$) after co-culture with MCAM-overexpressing hMSC compared to control. There were no significant qualitative differences in the formation of granulocytic (CFU-G), monocytic (CFU-M) or erythrocytic (BFU-E) lineages (Figure 3C).

MCAM knockdown induces RhoA activation

Since MCAM knockdown hMSC showed reduced migratory potential (Figure 1H) and an altered morphology associated with cell enlargement and stress fiber formation (*Online Supplementary Figure S4B*), we tested whether this could be mediated via the small GTPase RhoA. Therefore, we performed pull-down experiments to isolate active GTP-bound RhoA. As shown in *Online Supplementary Figure S4A*, MCAM knockdown hMSC exhibited a higher level of active RhoA compared to pLKO.1-control hMSC (1.47-fold). MCAM-overexpressing hMSC showed a decrease in the level of active RhoA compared to IRES-GFP-control hMSC (0.65-fold) indicating that MCAM expression modulates RhoA activity. To test whether we could pharmacologically mimic the morphological and migratory phenotype of MCAM knockdown in hMSC, transduced hMSC were treated with 10 μM Nocodazole, a known RhoA activator.[23] As shown in *Online Supplementary Figure S4B*, Nocodazole treatment of hMSC resulted in stress formation and impaired migration (*Online Supplementary Figure S4C*) in MCAM KD hMSC, MCAM overexpressing hMSC as well as in control hMSC. These data indicate that MCAM expression in hMSC modulates RhoA activity.

Discussion

During the last two decades, the characterization of the microenvironment regulating the fate of hematopoietic stem and progenitor cells (HSPC) has been an area of active research. Recent reports show that the cellular composition of the stem cell niche is more complex[24] than initially described.[25] Several groups have identified subsets of MSC populations as potential organizers of the hematopoietic microenvironment.[2,19,26-29] Sacchetti *et al.* found a population of self-renewing $CD45^-/MCAM^+$ perivascular osteoprogenitors in the adult human bone marrow.[1] Furthermore, Tormin *et al.* recently reported that MCAM expression correlated with its *in situ* localization.[10] Both reports suggest that $MCAM^+$ cells are an important component of the niche microenvironment. However, the functional relevance of MCAM in primary human MSC has not yet been described.

Recent studies of human term placenta-derived MSC demonstrated that MCAM expression correlated with the osteogenic differentiation potential of MSC.[30] Consistent with these data, we found that MCAM expression strongly affected the osteogenic differentiation potential of hMSC (Figure 1A and B). Our data suggest that MCAM knockdown also affected the potential for differentiating into an adipogenic lineage (Figure 1A and C). This suggests a global change in the intracellular signaling pathways relevant to differentiation.[31] In addition to the effect of MCAM on the differentiation potential of MSC, several lines of evidence suggest that MCAM expression also influences proliferation potential.[25-27] This was demon-

Table 1. Proportion of lineage specific subsets in the different compartments (non-adherent, phase-bright, phase-dim) after five days of co-culture with MCAM-KD or MCAM-GFP hMSC. pLKO.1 (for MCAM-KD) and IRES-GFP (for MCAM-GFP) transduced hMSC served as controls. MCAM overexpression maintained CD34 expression and the proportion of $CD34^+CD133^+$ HSPC compared with MCAM-KD in hMSC. MCAM-KD in hMSC resulted in an increased commitment into myeloid and lymphoid lineage.

Lineage	Compartment	Marker	MCAM-KD (n-fold to control)	MCAM-GFP (n-fold to control)
HSPC				
	Non-adherent	$CD34^+$	0.8±0.06	1.2±0.13
		$CD34^+CD133^+$	0.6±0.04	1.3±0.15
	Phase-bright	$CD34^+$	0.7±0.04	1.3±0.04
		$CD34^+CD133^+$	0.7±0.03	1.4±0.15
	Phase-dim	$CD34^+$	0.8±0.05	1.2±0.05
		$CD34^+D133^+$	0.5±0.07	1.3±0.10
Lymphoid differentiation				
	Non-adherent	$CD34^-CD10^+$	2.1±0.34	0.8±0.06
		$CD34^-CD56^+$	1.9±0.26	0.8±0.10
	Phase-bright	$CD34^-CD10^+$	2.1±0.08	0.7±0.08
		$CD34^-CD56^+$	2.4±0.53	0.7±0.09
	Phase-dim	$CD34^-CD10^+$	1.6±0.17	0.8±0.08
		$CD34^-CD56^+$	1.4±0.13	0.7±0.05
Myeloid differentiation				
	Non-adherent	$CD34^-CD13^+$	1.3±0.04	0.9 0.06
		$CD34^-CD33^+$	1.3±0.05	0.9±0.03
	Phase-bright	$CD34^-CD13^+$	1.6±0.18	0.8±0.07
		$CD34^-CD33^+$	1.5±0.15	0.8±0.03
	Phase-dim	$CD34^-CD13^+$	1.6±0.08	0.7±0.07
		$CD34^-CD33^+$	1.4±0.14	0.8±0.07

HSC compartment: $CD34^+$, $CD34^+CD133^+$; lymphoid lineage: $CD34^-CD10^+$, $CD34^-CD56^+$; myeloid lineage: $CD34^-$, $CD13^+$, $CD34^-CD33^+$. Data show proportions of depicted cell populations divided by the values obtained with corresponding control cells of at least 4 donors as mean ± SEM.

strated using MCAM+ hMSC that were initially derived from clonogenic colony-forming fibroblasts (CFU-F).[9] We confirmed that MCAM knockdown impaired the proliferation of hMSC (Figure 1D and E). This functional observation was associated with G1/S-phase cell cycle arrest mediated by the upregulation of p21 expression, the reduced expression of CDK2 and Cyclin E (Figure 1F and G) and the adoption of a senescent-like phenotype (*data not shown*).

MCAM seems to be relevant to the cell motility of melanoma and endothelial cells.[20,21,32] In agreement with these findings, we demonstrate that MCAM expression also affects the migration of hMSC (Figure 1H). Luo *et al.* suggested a possible molecular mechanism whereby MCAM may be involved in cytoskeleton remodeling and cell migration. These authors demonstrated an association between MCAM and ERM proteins that further induced RhoA activation.[21] Other groups showed that inhibition of RhoA induces cell migration.[33] We found elevated RhoA activity in hMSC upon MCAM knockdown with induced stress fiber formation and reduced migration (*Online Supplementary Figure S4*).

It is well established that mesenchymal stromal cells and soluble factors support the maintenance and proliferation of HSPC through different adhesion molecules.[1,5,8,22,34-36] Several groups have described the involvement of adhesion proteins such as N-cadherin, CD44, CD29, NCAM, or Notch, in mediating a direct interaction with the cellular microenvironment that enhances HSPC self-renewal.[1,4,37-41] We found that MCAM overexpression in hMSC led to reduced CD29 expression. In line with our findings, Walenda *et al.* reported that siRNA-mediated CD29 knockdown in hMSC resulted in enhanced numbers of more primitive HSPC after co-culture with these hMSC.[42] Furthermore, we demonstrate that MCAM knockdown resulted in an increased CD29 expression on hMSC, and an enhanced proliferation of HSPC associated with a loss of the more immature HSPC (Figure 2, Table 1). Long-term co-culture of HSPC with MCAM KD hMSC demonstrated a reduced capacity to facilitate the growth of cobblestone-forming HSPC, whereas a significantly higher number of CAFC (Figure 3A) and CFU frequency (Figure 3B) was found in co-culture with hMSC overexpressing MCAM. MCAM overexpression on hMSC maintained the immature cell fraction of HSPC that has a slower proliferation rate and is characterized by CD34 and CD133 expression (Figure 2). As we showed previously, the CD34+CD133+ cell fraction constitutes the HSPC subset with the highest marrow reconstitution potential in an NOD/SCID xenotransplantation model.[41]

Here we show that MCAM has a crucial role in regulating the proliferation and maintenance of HSPC during *ex vivo* co-culture (Figures 2 and 3 and *Online Supplementary Figure S2*). MCAM knockdown resulted in a severe impairment of the supportive function of hMSC for HSPC, whereas MCAM overexpression supported the maintenance of HSPC independent of MSC fate.

In contrast to our results, Sharma and co-workers demonstrated an increased support of HSPC by placenta-derived MCAM negative hMSC.[43] These placenta-derived hMSC are initially MCAM positive. After culture in a 3-dimensional hydrogel-based matrix, which constitutes hypoxic conditions, MCAM expression is lost. Concordantly, Tormin *et al.* demonstrated that MCAM is down-regulated under hypoxic conditions.[10] Furthermore,

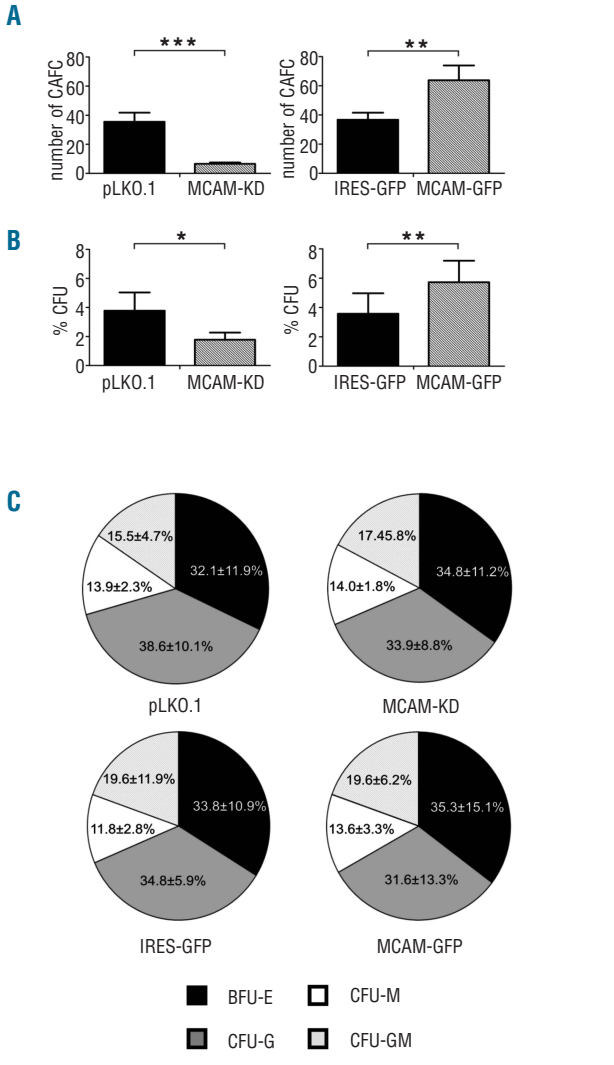

Figure 3. MCAM expression is important for the long-term maintenance of HSPC. (A) HSPC derived from umbilical cord blood were cultured on hMSC that stably expressed MCAM shRNA or that overexpressed MCAM in long-term initiating cell assays (LTC-IC). Colony-area forming cells (CAFC) were counted after four weeks. Mean±SEM for 4 independent hMSC and HSPC donors is shown (P<0.01; ***P<0.001; paired Student's t-test). (B) Clonal growth of CAFC derived HSPCs after four weeks of co-culture on MCAM knockdown or MCAM-overexpressing hMSC monolayer was analyzed in a secondary colony-forming cell assay (CFU) for an additional 14 days. The frequency of CFUs was determined as the total number of colonies relative to the number of CD45+ cells. Mean±SEM for 4 donors is shown (*P<0.05; **P<0.01; paired Student's t-test). (C) Proportion of erythroid, granulocytic, or monocytic lineages in the secondary CFU-assay (Cochran-Mantel-Haenszel test). Mean±SEM for 4 donors is shown. BFU-E: burst-forming unit-erythroid: CFU-G: colony-forming unit-granulocyte; CFU-M: colony-forming unit-macrophage; CFU-GM: colony-forming unit-granulocyte macrophage.**

it was shown by others and our group that oxygen tension causes selective modification of hematopoietic cell and mesenchymal stromal cell interactions in co-culture systems as well as influence HSPC metabolism.[44-46] Thus, the observed differences between Sharma *et al.* and our data in HSPC supporting capacity of hMSC are likely due to the different culture conditions used. Further studies are required to clarify the influence of hypoxia in our model system. Altogether these findings provide further evidence

for the importance of MCAM in supporting HSPC. Furthermore, previous reports have shown that MCAM is down-regulated in MSC after several passages as well as during aging and differentiation.[19,47]

Interestingly, MCAM overexpression in hMSC enhanced the adhesion of HSPC to MSC and it further supported the migration of HSPC (*Online Supplementary Figure S2A and B*). Previous reports show that monocyte transendothelial migration involves a heterophilic interaction between MCAM on endothelial cells and an unidentified counterpart on monocytes.[12] The current study also suggests that MCAM acts through a direct cell-cell interaction (*Online Supplementary Figure S2A*). However, HSPC do not express MCAM (*Online Supplementary Figure S5*), so the putative interaction partner of MCAM on HSPC remains elusive. Instead of direct receptor interactions, functional changes after MCAM expression may also account for the observed maintenance of HSPC in co-culture with hMSC. Because the SDF-1/CXCR4 axis has a key role in the homing and mobilization of HSPC, as well as having a positive impact on the maintenance of HSPC, we tested whether SDF-1 is regulated upon MCAM expression.[48-50] However, we observed no effect of MCAM on SDF-1 expression or secretion by hMSC (*data not shown*). The clinical relevance of our findings is that the functional properties of hMSC are clearly related to MCAM expression. Tripodo *et al*. demonstrated an involvement of MCAM$^+$ osteoprogenitors in the advanced stages of myelofibrosis.[51] Furthermore, MCAM has an aberrant expression on several tumor cell types[52,53] Thus, MCAM may act as a binding partner for circulating tumor cells that have been shown to hijack the bone marrow microenvironment. Therefore, interfering with the action of MCAM on MSC, or other skeletal progenitor cells and tumor cells may be an option for reducing the homing of tumor cells to the bone marrow and their subsequent metastatic spread.

We showed that MCAM expression in hMSC supports the growth of hematopoietic progenitors. Further studies will be required to unravel the intracellular consequences in hMSC upon MCAM knockdown or overexpression as well as to establish the *in vivo* relevance of MCAM expression for the maintenance of hematopoietic stem cells. Furthermore, studies should analyze whether MCAM-overexpressing hMSC are suitable to expand HSPC from cord blood products to facilitate better engraftment after allogeneic transplantation, particularly in adult patients where the quantity of HSPC available in single cord blood units might be a limiting factor.

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Authorship and Disclosures

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